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Heat Shock Protein Vaccines Against Cancer

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Summary: Vaccination of mice with heat shock proteins (HSPs) derived from a tumor makes the mice resistant to the tumor from which the HSP was obtained. This phenomenon has been demonstrated with three HSPs—gp96, hsp90, and hsp70. Vaccination with HSPs also elicits antigen-specific cytotoxic T lymphocytes (CTLs). The specific immunogenicity of HSPs derives apparently, not from the HSPs per se, but from the peptides bound to them. These observations provide the basis for a new generation of vaccines against cancer. The HSP-based cancer vaccines circumvent two of the most intractable hurdles to cancer immunotherapy. One of them is the possibility that human cancers, like cancers of experimental animals, are antigenically distinct. The prospect of identification of immunogenic antigens of individual cancers from patients is daunting to the extent of being impractical. The observation that HSPs chaperone antigenic peptides of the cells from which they are derived circumvents this extraordinary hurdle. Second, most current approaches to cancer immunotherapy focus on determining the CTL-recognized epitopes of cancer cell lines. This approach requires the availability of cell lines and CTLs against cancers. These reagents are unavailable for an overwhelming proportion of human cancers. In contrast, the HSP-based vaccines do not depend on the availability of cell lines or CTLs nor do they require definition of the antigenic epitopes of cancer cells. These advantages, among others, make HSPs attractive and novel immunogens against cancer. Key Words: Gp96—hsp90—hsp70—Infectious diseases—Immunity.

Immunogenicity of cancers was first demonstrated convincingly in methylcholanthrene-induced fibrosarcomas of inbred mice (1–4). Immunization of mice with a given tumor renders the mice resistant to that particular tumor, but not to another tumor, even though both tumors may be induced by the same carcinogen and be of the same histological origin (3–6). Tumors induced by a variety of carcinogens and even spontaneous tumors, in several inbred strains of mice, rats, and guinea pigs, as well as tumors of diverse histological origins, display individually specific antigenicity (7). The individually distinct tumor rejection antigens of these tumors are the prototypical tumor antigens and many of the cardinal principles of cancer immunology derive from their study.

Identity of these antigens has been the focus of much unsuccessful scrutiny over the years and they remain biochemically uncharacterized to date. Three major approaches to their identification have been attempted so far. (a) Serological approach: whereby tumor-specific antigens that elicit antibody response in autologous or syngeneic systems have been sought. Only a very small number of such antigens have been detected and characterized so far

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(8–10). (b) T-cell approach: whereby antigens defined by tumor-specific cytotoxic T-lymphocytes (CTLs) have been sought. However, methods to identify CTL epitopes have not been available until very recently. The work of Boon and his colleagues (11–13) is the only successful example of such an effort and has resulted in characterization of CTL-recognized tumor antigens on P815 mouse mastocytoma and human melanomas. However, the ability these antigens to *protect* against tumor challenges remains undemonstrated. (c) Transplantation approach: whereby tumor-derived antigens are identified by their ability to vaccinate mice against subsequent tumor challenges. This approach has resulted in identification of heat shock proteins (HSPs) as being able to provide tumor-specific immunity to the tumors from which HSPs are obtained but not against other tumors (9,14–16, H.U. and P.K.S., unpublished observations; A. M. Feldweg and P.K.S. unpublished observations). HSPs are quintessential self-antigens and their tumor-specific immunogenicity is a paradox, whose resolution lies perhaps in the observation that they chaperone a diverse array of antigenic peptides that elicit immunity (17,18). Studies reported here show that in addition to conferring protective tumor immunity, immunization with HSPs also elicits antigen-specific major histocompatibility complex (MHC) class I–restricted CTL response against the tumors or virus-infected cells from which the HSPs are purified.

MATERIALS AND METHODS

Mice and Antibodies

BALB/cJ mice (viral antigen-free) were obtained from Jackson Laboratories and were maintained in the virus-free mouse facilities at Mount Sinai School of Medicine. Antibodies to gp96 (anti-GRP94, SPA-850, clone 9G10) and hsp70 (anti-HSP72/73, SPA-820, clone N27F3-4) were purchased from StressGen. Antibody to hsp90 (anti-HSP90, MA3-011, clone 3G3) was obtained from Affinity BioReagents.

Purification of GP96, HSP90, and HSP70

Gp96, hsp90, and hsp70 were purified simultaneously from Meth A sarcoma cells, by a combination of published methods (14,15,19) and some modifications (H.U. and P.K.S., unpublished observations).

SDS–Polyacrylamide Gel Electrophoresis (PAGE) and Protein Blotting

Proteins were resolved on SDS-PAGE, electrophoresed, blotted to nitrocellulose, and probed with appropriate antibodies, as described (14).

Generation and Assay of CTL Activity

Mice were injected subcutaneously with HSP preparations, twice, 10 days apart. Ten days later spleen cells were stimulated in vitro with the cells used for isolation of HSPs, employing a mixed tumor-lymphocyte culture (MLTC) method described recently (20). Briefly, 8×10^6 immune spleen cells were restimulated with 4×10^4 irradiated tumor cells in 3 ml RPMI 10% fetal calf serum. In certain experiments 33% secondary mixed lymphocyte culture-supernatant was included in the culture medium as a source of T-cell growth factors. Six days later the cultures were tested for cytotoxicity in a 4-h ^{51}Cr-release assay. The spontaneous ^{51}Cr-release of the targets was regularly <20%. For MHC class I blocking, 10 times concentrated hybridoma supernatant of K-44 was added to the test at a final concentration of 12.5%.

Tumor Rejection Assays

Mice were immunized with proteins or tumors twice at weekly intervals and challenged by intradermal injections of live tumor cells 1 week after the last immunization. A high viability of tumor cells (>98%) is an important prerequisite for reproducible results.

RESULTS AND DISCUSSION

Tumor-Specific Immunogenicity of Cognate HSP Preparations

BALB/cJ mice were vaccinated with apparently homogeneous preparations of gp96 (6 μg) obtained from the Meth A sarcoma or normal tissues. Control mice were vaccinated with phosphate buffered saline (PBS). All mice were challenged with 100,000 Meth A cells. The dose of gp96 and the level of challenge was determined after optimization of vaccination with different doses of gp96 and challenge

with different numbers of tumor cells (H.U. and P.K.S., unpublished observations). It was observed (Fig. 1) that tumors grew progressively in all five mice vaccinated with PBS, most of the mice (three of four) vaccinated with gp96 derived from normal liver and spleen, but in none of the four mice vaccinated with Meth A–derived gp96. The Meth A gp96–immunized mice remained disease free for at least four additional months, at which time they were sacrificed. This immunity was tumor specific, as antigenically distinct methylcholanthrene-induced fibrosarcomas CMS4 and CMS5 grew progressively in these mice. Similar observations have been made with other HSPs such as hsp90, which is the cytosolic counterpart of gp96 and hsp70 (H.U. and P.K.S., unpublished observations).

The phenomenon of tumor-specific immunogenicity of cognate HSPs has also begun to be tested in UV-induced fibrosarcomas of C3H He/N mice. We have chosen the antigenically distinct sarcomas UV6138 and UV6139 (20) for our studies. As these tumors are regressor tumors, it is not possible to do transplantation assays with these tumors in the same manner as with progressor tumors. It is possible, however, to sublethally irradiate the mice after vaccination and immediately prior to challenge; under these conditions a challenge with a regressor tumor results in a progressive growth of the tumor in unimmunized mice, but not in mice immunized with the cognate tumor. This immunity is tumor specific. Preliminary studies with gp96 isolated from the sarcoma UV6138 suggest a protective role for this HSP against a cognate challenge (S.J., N.E.B., and P.K.S., unpublished observations).

These observations confirm and extend our original observations of tumor-specific immunogenicity of HSPs derived from cognate tumor cells (9,14–16).

Vaccination with HSPs Elicits Antigen-Specific MHC Class I–Restricted CTLs Against Cognate Targets

We have proposed that HSPs elicit tumor-specific immunity not because they are antigenic per se, but because they are carriers of antigenic peptides (17,21). Association of peptides with gp96 and hsp70 has also been shown experimentally by us (18) and Rothman and colleagues (22,23). The gp96 HSP is among the major components of the lumen of the endoplasmic reticulum, which is also the sight of charging of MHC class I molecules with peptides. Further, gp96 binds ATP and is an ATPase and would therefore be an excellent candidate for accepting peptides from the peptide transporter TAP proteins and transferring them to MHC class I molecules. Should this indeed be true, gp96 molecules would be expected to associate with the entire antigenic repertoire of any cell, be it a normal cell, a tumor cell, or a virally infected cell. We have begun to examine this proposition by vaccinating mice with gp96 from influenza-infected cells or SV40-transformed cells, and testing the MLTCs from spleens of these mice for antigen-specific MHC class I–restricted CTL activity.

Preliminary experiments reveal that mice immunized with gp96 preparations indeed develop CTL activity against the cognate targets (Table 1) (N.B. and P.K.S., unpublished observations). Thus, MLTCs generated from spleens of mice immunized with gp96 from the flu-NP–transfected BALB/c cell line BC/NP (obtained from Dr. Eli Gilboa, Duke University) show CTL reactivity against BC/NP but not a non–NP expressing syngeneic line. Similarly, MLTCs generated from spleens of mice immunized with gp96 from the SV40-transformed C57BL/6 line SVB6 (obtained from Dr. Satvir Tevethia, Pennsyl-

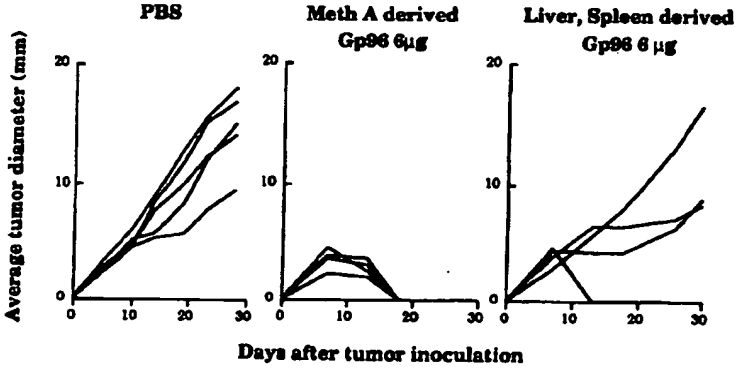

FIG. 1. Tumor-specific immunogenicity of gp96 preparations derived from the BALB/cJ sarcoma Meth A. Mice were immunized with phosphate buffered saline or 6 μg gp96 derived from Meth A or an equal quantity of gp96 derived from liver and spleens of normal BALB/cJ mice. All mice were challenged with 100,000 Meth A cells. Each line represents the kinetics of tumor growth in a single mouse.

TABLE 1. *Vaccination of mice with GP96 preparations elicits antigen-specific major histocompatibility complex (MHC) class I restricted cytotoxic T-lymphocyte reactivity against the cells from which gp96 is isolated*

Source of gp96	Target	% MHC class I restricted killing E/T ratio of 25:1
UV-6138	UV-6138	11.2
	UV-6139	1.5
BC/NP	BC/NP	38.0
	UV-5117	0.0
SVB6	SVB6	31.2
	MCA	0.0

E/T, effector-to-target.

vania State University) show CTL reactivity against SVB6 but not a non–SV40 transformed syngeneic line. Similar results are seen with mice immunized with gp96 derived from the UV-induced fibrosarcoma 6138.

HSPs as Vaccines Against Cancer

Our results demonstrate that vaccination with HSPs elicits tumor immunity and an antigen-specific $CD8^+$ CTL response. This observation has a direct bearing on immunotherapy of cancer. Vaccination with HSPs confers a number of unique advantages over other methods of vaccination against cancer and these are discussed.

One of the major conceptual difficulties in cancer immunotherapy has been the possibility that human cancers, like cancers of experimental animals, are antigenically distinct. Clearly, there is some recent evidence for existence of common human tumor antigens (24,25), and this augurs well for prospects of cancer immunotherapy. Nonetheless, in light of the overwhelming evidence from experimental and human systems, it is reasonable to assume that at the very least, human tumors would show tremendous antigenic diversity and heterogeneity. The prospect of identification of the immunogenic antigens of individual tumors from cancer patients (or even of only several different types of immunogenic antigens in case the antigens are shared) is daunting to the extent of being impractical. The possibility that HSPs chaperone antigenic peptides from cells from which they are derived circumvents this hurdle.

Second, most current approaches to cancer immunotherapy focus on determining the CTL-recognized epitopes of cancer cell lines. These approaches require availability of cell lines and cloned CTLs against the cancers. These reagents cannot be generated for an overwhelming proportion of human solid cancers. Even for the extremely small proportion of human cancers for which these reagents may be available, it is nearly impossible to hope to identify the CTL-recognized epitopes within a patient's lifetime, with the current level of technology for identification of such epitopes. In contrast, the HSP-based vaccines do not depend on availability of cell lines or CTLs nor do they require definition of the antigenic epitopes of cancer cells.

The biochemical purity of HSP preparations is another advantage of vaccination with HSPs over other methods. Immunization with biochemically undefined tumor extracts inevitably carries the risk of inoculating the mice or patients with potentially transforming or immunosuppressive agents such as transforming DNA or transforming growth factor β (TGFβ). Immunization with purified HSP preparations eliminates these risks. Further, while it is conceivable that use of appropriate adjuvants may magnify the amplitude of this response, their use is not necessary for a vigorous response. (It is of interest in this regard that the use of Freund's adjuvant with gp96 abrogates rather than potentiates the antitumor response.)

HSPs as Vaccines Against Infectious Diseases

The ability of HSPs to elicit cellular immune response against virtually any antigens expressed by a cell makes them attractive vaccine candidates against infectious diseases as well. Particularly suitable are the diseases where the protective antigenic epitopes are undefined, or difficult to define, or highly variable. The human immunodeficiency viruses are an appropriate example of such an infection.

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REFERENCES

1. Gross L. Intradermal immunization of C3H mice against a sarcoma that originated in an animal of the same line. *Cancer Res* 1943;3:323–6.
2. Prehn R, Main JM. Immunity to methylcholanthrene-induced sarcomas. *J Natl Cancer Inst* 1957;18:769–78.
3. Klein G, Sjogren HO, Klein E, Hellstrom KE. Demonstration of resistance against methylcholanthrene-induced sarcomas in the primary autochthonous host. *Cancer Res* 1960; 20:1561–72.

4. Old LJ, Boyse EA, Clarke DA, Carswell EA. Antigenic properties of chemically induced tumors. *Ann NY Acad Sci* 1962;101:80–106.
5. Basombrío MA. Search for common antigenicities among 25 sarcomas induced by methylcholanthrene. *Cancer Res* 1970; 30:2458–62.
6. Globerson A, Feldman M. Antigenic specificity of benzo(a)pyrene induced sarcomas. *J Natl Cancer Inst* 1964;32: 1229–43.
7. Srivastava PK, Old LJ. Individually distinct transplantation antigens of chemically induced mouse tumors. *Immunol Today* 1988;9:78–83.
8. DeLeo AB, Shiku H, Takahashi T, John M, Old LJ. Cell surface antigens of chemically induced sarcomas of mouse. MuLV related antigens and alloantigens on cultured fibroblasts and sarcoma cells: description of a unique antigen on BALB/c Meth A sarcoma. *J Exp Med* 1977;146:1516–30.
9. Srivastava PK, Das MR. The serologically unique cell surface antigen of Zajdela ascitic hepatoma is also its tumor associated transplantation antigen. *Int J Cancer* 1984;33: 417–22.
10. Furukawa KS, Furukawa R, Real FX, Old LJ, Lloyd KO. A unique antigenic epitope of human melanoma is carried on the common melanoma glycoprotein gp95/p97. *J Exp Med* 1989;169:585–90.
11. Van den Eynde B, Lethe B, VanPel A, DePlaen E, Boon T. The gene coding for a major tumor rejection antigen of tumor P815 is identical to the normal gene of syngeneic DBA/2 mice. *J Exp Med* 1991;173:1373–84.
12. Traversari C, van der Bruggen P, Luescher IF, et al. A nonapeptide encoded by MAGE-1 is recognized on HLA A1 by cytolytic T lymphocytes directed against tumor antigen MZ2-E. *J Exp Med* 1992;176:1453–7.
13. Boon T. Towards a genetic analysis of tumor rejection antigens. *Adv Cancer Res* 1992;58:177–210.
14. Srivastava PK, DeLeo AB, Old LJ. Tumor rejection antigens of chemically induced tumors of inbred mice. *Proc Natl Acad Sci USA* 1986;83:3407–807.
15. Ullrich SJ, Robinson EA, Law LW, Willingham M, Appella E. A mouse tumor-specific transplantation antigen is a heat-shock related protein. *Proc Natl Acad Sci USA* 1986;83: 3121–5.
16. Palladino MA, Srivastava PK, Oettgen HF, DeLeo AB. Expression of a shared tumor-specific antigen by two chemically induced BALB/c sarcomas. *Cancer Res* 1987;47: 5074–9.
17. Srivastava PK, Maki RG. Stress-induced proteins in immune response to cancer. *Curr Top Microbiol Immunol* 1991;167: 109–23.
18. Li Z, Srivastava PK. Tumor rejection antigen gp96/grp94 is an ATPase: implications for antigen presentation and protein folding. *EMBO Journal* (in press).
19. Welch WJ, Feramisco JR. Rapid purification of mammalian 70,000-d stress proteins: affinity of proteins for nucleotides. *Mol Cell Biol* 1985;5:1229–37.
20. Ward PL, Koeppen HK, Hurteau H, Rowley DA, Schreiber H. Tumor antigens defined by cloned immunological probes are highly polymorphic and are not detected on autologous normal cells. *J Exp Med* 1990;170:217–32.
21. Srivastava PK. Peptide-binding heat shock proteins of the endoplasmic reticulum: role in antigen presentation and in immune response to cancer. *Adv Cancer Res* (in press).
22. Flynn GC, Chappell TG, Rothman JE. Peptide binding and release by proteins implicated as catalysts of protein assembly. *Science* 1989;245:385–8.
23. Flynn GC, Pohl J, Flocco MT, Rothman JE. Peptide-binding specificity of the molecular chaperone BiP. *Nature* 1991; 353:726–30.
24. Kawakami Y, Zakut R, Topalian SL, Stotter H, Rosenberg SA. Shared human melanoma antigens. Recognition by tumor-infiltrating lymphocytes in HLA-A2.1–transfected melanomas. *J Immunol* 1992;148:638–43.
25. Darrow TL, Slingluff CL, Siegler HF. The role of HLA Class I antigens in recognition of melanoma cells by tumor-specific T lymphocytes: evidence for shared tumor antigens. *J Immunol* 1989;142:3329.